

Chlorocillium sinense sp. nov. (Clavicipitaceae) and *Calcarisporium guizhouense* sp. nov. (Calcarisporiaceae) in Hypocreales from China

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Abstract

Two new species, *Chlorocillium sinense* and *Calcarisporium guizhouense*, isolated from a spider and fruiting body of *Cordyceps* sp., are introduced. Morphological comparisons and phylogenetic analyses based on multigene datasets (ITS+LSU+RPB2+tef-1alpha) support the establishment of the new species. A combined dataset of ITS, LSU, RPB2, and tef-1alpha showed the taxonomic placement of *Chlorocillium* in Clavicipitaceae for the first time. *Pseudometarhizium* is regarded as a synonym of *Chlorocillium* and two *Pseudometarhizium* species are transferred into the latter based on the phylogenetic analysis and morphological characteristics.

Key words: Entomopathogenic fungi, morphology, phylogenetic analysis, Sordariomycetes, taxonomic placement



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Introduction

During a survey of fungi associated with insects and spiders from Southwest China, a new spider-associated species and a new hyperparasitic species were found. The morphological characteristics and BLAST results revealed that the new collections belong to *Chlorocillium* and *Calcarisporium*. The genus *Chlorocillium* Zare & W. Gams was proposed to accommodate the species in *Verticillium* section *Albo-erecta*, and *Chlorocillium griseum* (Petch) Zare & W. Gams was described as the type species (Zare and Gams 2016). The typical characteristics of *Chlorocillium* are its slow-growing and greenish-ochraceous colony, conidiophores short with meagre whorls of phialides and fusiform conidia in chains (Zare and Gams 2016). The genus comprises the type species and two other species, *C. guerinae* Y.P. Tan et al. and *C. montefioreae* Y.P. Tan et al. (Tan and Shivas 2023, 2024).

The genus *Calcarisporium* Preuss was introduced with *C. arbuscula* Preuss as the type species (Preuss 1851). The typical characteristics of *Calcarisporium* are its verticillate conidiophores, holoblastic conidiogenous cells, polyblastic, sympodial conidiation and ovoid to ellipsoidal conidia (Hughes 1951, de Hoog

1974). Sun et al. (2017) accepted six species i.e., *C. acerosum* Matsush., *C. arbuscula*, *C. cordycipiticola* Jing Z. Sun et al., *C. ovalisporum* (Petch) de Hoog, *C. phaeopodium* Somrith. & E.B.G. Jones and *C. xylariicola* Jing Z. Sun et al. Later, Zhu et al. (2023) introduced *C. yuanyangense* H. Yu bis & J.Y. Zhu.

In our phylogenetic analyses of combined ITS, LSU, *RPB2* and *tef1-α* sequences, *Chlorocillium* species clustered in Clavicipitaceae (Hypocreales, Hypocreomycetidae) with strong statistical support. Thus, we propose that *Chlorocillium* belongs to the family Clavicipitaceae. The new collections are phylogenetically and morphologically distinct from other known species. Thus, we introduce *Chlorocillium sinense* sp. nov. and *Calcarisporium guizhouense* sp. nov. Besides, *Pseudometarhizium* species groups with *Chlorocillium* s. str., and thus, we propose to synonymise *Pseudometarhizium* under *Chlorocillium* based on the phylogenetic analyses.

Materials and methods

Specimen collection and identification

The specimens were collected from Monkey-Ear Tiankeng (27°5'12.138"N, 107°0'48.42"E), Kaiyang County, Guiyang and Mayao River Valley (26°22'8.3748"N, 107°23'16.96"E), Duyun City, Qiannan Buyei and Miao Autonomous Prefecture, Guizhou Province, on 19 July 2023 and 1 May 2022. The samples were placed in an ice box and brought to the laboratory. Specimens were preserved in the refrigerator at 4 °C until further processing. The surface of each arthropod body was rinsed with sterile water, followed by sterilization with 75% ethanol for 3–5 s and rinsing again three times with sterilized water. After drying on sterilized filter paper, a piece of the synnemata, mycelium or sclerotia was cut from the specimen and placed on plates of potato dextrose agar (PDA) or PDA modified by the addition of 1% w/v peptone containing 0.1 g/l streptomycin and 0.05 g/l tetracycline (Chen et al. 2019). After fungal colonies emerged from the inoculated samples, a piece of mycelium from the colony edge was transferred onto new agar plates and cultured at 25 °C for 14 days under 12 h light/12 h dark conditions (Zou et al. 2010). The holotypes and ex-types were deposited at the Institute of Fungus Resources, Guizhou University (formally Herbarium of Guizhou Agricultural College; code, GZAC), Guiyang City, Guizhou, China. MycoBank numbers were obtained as outlined in MycoBank (<http://www.MycoBank.org>) (Crous et al. 2004).

Colony morphology was determined on PDA cultures incubated at 25 °C for 14 days and the growth rate, the presence of octahedral crystals and colony colors (surface and reverse) were observed. To investigate the microscopic characteristics, a little of the mycelia was picked up from the colony and mounted in lactophenol cotton blue or 20% lactic acid solution and the asexual morphological characteristics (e.g., conidiophores, phialides and conidia) were observed and measured using a Leica DM4 B microscope.

DNA extraction, polymerase chain reaction amplification and nucleotide sequencing

DNA extraction was carried out using a fungal genomic DNA extraction kit (DP2033, BioTeke Corporation) according to Liang et al. (2011). The extracted DNA was stored at -20 °C. Polymerase chain reaction (PCR) was used to amplify

genetic markers using the following primer pairs: ITS4/ITS5 for the internal transcribed spacer (ITS) region (White et al. 1990), LR0R/LR5 for 28s large subunit ribosomal (LSU) (Vilgalys and Hester 1990), fRPB2-5F/fRPB2-7cR for RNA polymerase II second largest subunit (*RPB2*) (Liu et al. 1999) and 983F/2218R for translation elongation factor 1 alpha (*tef-1α*) (Castlebury et al. 2004). The thermal cycle of PCR amplification for these phylogenetic markers was set up following the procedure described by Chen et al. (2021). PCR products were purified and sequenced at Sangon Biotech (Shanghai) Co. All newly generated sequences were deposited in GenBank and accession numbers were obtained (Table 1).

Table 1. List of strains and GenBank accession numbers of sequences used in this study.

Species	Strain No.	GenBank Accession No.			
		ITS	LSU	<i>RPB2</i>	<i>tef-1α</i>
<i>Aciculosporium oplismeni</i>	MAFF 246966	LC571760	LC571760	LC572054	LC572040
<i>Aciculosporium take</i>	MAFF 241224	LC571753	LC571753	LC572048	LC572034
<i>Aciculosporium take</i>	TNS-F-60465	LC571755	LC571756	LC572049	LC572035
<i>Akanthomyces aculeatus</i>	HUA 772	KC519371	–	–	KC519366
<i>Aschersonia confluens</i>	BCC 7961	JN049841	DQ384947	DQ452465	DQ384976
<i>Aschersonia placenta</i>	BCC 7869	JN049842	EF469074	EF469104	EF469056
<i>Atkinsonella hypoxylon</i>	B4728	–	–	KP689514	KP689546
<i>Balansia epichloe</i>	A.E.G. 96-15a	–	–	EF468908	EF468743
<i>Balansia henningsiana</i>	A.E.G. 96-27a	JN049815	AY545727	DQ522413	AY489610
<i>Balansia pilulaeformis</i>	A.E.G. 94-2	–	AF543788	DQ522414	DQ522319
<i>Bionectria ochroleuca</i>	AFTOL-ID 187	–	DQ862027	DQ862013	DQ862029
<i>Bionectria vesiculosa</i>	HMAS 183151 ^T	HM050304	HM050302	–	–
<i>Calcarisporium arbuscula</i>	CBS 221.73	AY271809	–	–	–
<i>Calcarisporium arbuscula</i>	CBS 900.68	KT945003	KX442598	KX442597	KX442596
<i>Calcarisporium cordycipiticola</i>	CGMCC 3.17905	KT944999	KX442599	KX442594	KX442593
<i>Calcarisporium cordycipiticola</i>	CGMCC 3.17904	KT945001	KX442604	KX442607	KX442605
<i>Calcarisporium guizhouense</i>	DY05041^T	PP124948	PP133530	–	PP146564
<i>Calcarisporium guizhouense</i>	DY05042	PP809658	PP809662	–	PP823899
<i>Calcarisporium xylariicola</i>	HMAS 276836 ^T	KX442603	KX442601	KX442606	KX442595
<i>Calonectria ilicicola</i>	CBS 190.50	GQ280605	GQ280727	KM232307	AY725726
<i>Cephalosporium curtipes</i>	CBS 154.61	AJ292404	AF339548	EF468947	EF468802
<i>Chlorocillium griseum</i>	CBS 387.73 ^T	KU382150	KU382218	–	–
<i>Chlorocillium griseum</i>	RCEF6632	MW031768	MW084342	MW091329	MW091327
<i>Chlorocillium guerinae</i>	BRIP 72680a ^T	OR750699	OR731505	OR737788	OR737799
<i>Chlorocillium guerinae</i>	BRIP 72666a	OR750701	OR731507	OR737790	OR737801
<i>Chlorocillium guerinae</i>	BRIP 72668a	OR750702	OR731508	OR737791	OR737802
<i>Chlorocillium montefioreae</i>	BRIP 70299a ^T	PP420202	PP415875	PP438395	PP438400
<i>Chlorocillium sinense</i>	KY07181^T	PP768154	PP768156	PP766578	PP766580
<i>Chlorocillium sinense</i>	KY07182	PP768155	PP768157	PP766579	PP766581
<i>Claviceps fusiformis</i>	ATCC 26019	JN049817	U17402	–	DQ522320
<i>Claviceps purpurea</i>	GAM 12885	U57669	AF543789	DQ522417	AF543778
<i>Claviceps purpurea</i>	S.A. cp11	–	EF469075	EF469105	EF469058
<i>Clonostachys rosea</i>	GJS90-227	–	AY489716	–	AY489611
<i>Collarina aurantiaca</i>	FMR 11134	KJ807178	KJ807181	–	–
<i>Collarina aurantiaca</i>	FMR 11784	KJ807177	KJ807180	–	–
<i>Conoideocrella luteorostrata</i>	NHJ 11343	JN049859	EF468850	–	EF468801
<i>Conoideocrella luteorostrata</i>	NHJ 12516	JN049860	EF468849	EF468946	EF468800
<i>Conoideocrella tenuis</i>	NHJ 6293	JN049862	EU369044	EU369087	EU369029
<i>Cocoonihabitatus sinensis</i>	HMAS254523 ^T	KY924870	KY924869	–	–
<i>Cocoonihabitatus sinensis</i>	HMAS254524	MF687395	MF687396	–	–

Species	Strain No.	GenBank Accession No.			
		ITS	LSU	<i>RPB2</i>	<i>tef-1α</i>
<i>Corallocyostroma ornithocopreoides</i>	WAC 8705	–	–	LT216620	LT216546
<i>Cordyceps brongniartii</i>	BCC16585	JN049867	JF415967	JF415991	JF416009
<i>Cordyceps militaris</i>	OSC93623	JN049825	AY184966	–	DQ522332
<i>Dactylonectria alcacerensis</i>	CBS 129087	JF735333	KM231629	–	JF735819
<i>Dussiella tuberiformis*</i>		–	–	JQ257020	JQ257027
<i>Elaphocordyceps ophioglossoides</i>	NBRC 106332	JN943322	JN941409	–	–
<i>Elaphocordyceps paradoxa</i>	NBRC 106958	JN943324	JN941411	–	–
<i>Ephelis japonica</i>	CBS 236.64	MH858427	–	–	–
<i>Ephelis japonica</i>	Eph.oryzae	AB038564	–	–	–
<i>Ephelis tripsaci</i>	CBS 857.72 ^T	NR_153997	NG_059240	–	–
<i>Epichloe elymi</i>	C. Schardl 760	–	AY986924	–	AY986951
<i>Epichloe typhina</i>	ATCC 56429	JN049832	U17396	DQ522440	AF543777
<i>Flammocladiella aceris</i>	CPC 24422	KR611883	KR611901	–	–
<i>Fusarium circinatum</i>	CBS 405.97	U61677	–	JX171623	KM231943
<i>Fusarium sublunatum</i>	CBS 189.34 ^T	HQ897830	KM231680	–	–
<i>Gelasinospora tetrasperma</i>	AFTOL-ID 1287	–	DQ470980	DQ470932	DQ471103
<i>Haptocillium sinense</i>	CBS 567.95	AJ292417	AF339545	–	–
<i>Helicocollum surathaniensis</i>	BCC 34463	–	KT222328	–	KT222336
<i>Helicocollum surathaniensis</i>	BCC 34464 ^T	–	KT222329	–	KT222337
<i>Heteroepichloe bambusae</i>	Ba-01	AB065426	–	–	–
<i>Heteroepichloe bambusae</i>	Bo-01	AB065428	–	–	–
<i>Heteroepichloe sasae</i>	<i>E.sasae</i> -H	AB065432	–	–	–
<i>Heteroepichloe sasae</i>	<i>E.sasae</i> -N	AB065431	–	–	–
<i>Hydropisphaera erubescens</i>	ATCC 36093	–	AF193230	AY545731	DQ518174
<i>Hydropisphaera lutea</i>	ATCC 208838	–	AF543791	DQ522446	AF543781
<i>Hydropisphaera peziza</i>	GJS92-101	–	AY489730	–	AY489625
<i>Hydropisphaera rufa</i>	DAOM JBT1003	JN942883	JN938865	–	–
<i>Hypocrea americana</i>	AFTO -ID 52	DQ491488	AY544649	–	DQ471043
<i>Hypomyces polyporinus</i>	ATCC 76479	–	AF543793	–	AF543784
<i>Keithomyces carneus</i>	CBS 239.32	NR_131993	NG_057769	EF468938	EF468789
<i>Lecanicillium attenuatum</i>	CBS 402.78	AJ292434	AF339565	EF468935	EF468782
<i>Lecanicillium lecanii</i>	CBS 101247	JN049836	KM283794	KM283859	DQ522359
<i>Lecanicillium psalliotae</i>	CBS 367.86	–	KM283800	–	KM283823
<i>Marquandomyces marquandii</i>	CBS 182.27	NR_131994	EF468845	EF468942	EF468793
<i>Marquandomyces</i> sp.	CBS 127132	MT078882	MT078857	MT078922	–
<i>Metapochonia bulbillosa</i>	CBS 145.70	MH859529	AF339542	EF468943	EF468796
<i>Metapochonia gonioides</i>	CBS 891.72	AJ292409	AF339550	DQ522458	DQ522354
<i>Metapochonia rubescens</i>	CBS 464.88 ^T	–	AF339566	EF468944	EF468797
<i>Metapochonia sulchlasporia</i>	CBS 251.83	NR_154139	MH873311	–	KJ398790
<i>Metarhiziosis microspora</i>	CEHS133a	EF464589	EF464571	–	–
<i>Metarhiziosis microspora</i>	INEHS133a	EF464583	EF464572	–	–
<i>Metarhizium anisopliae</i>	ARSEF 7487	HQ331446	–	DQ468370	DQ463996
<i>Metarhizium anisopliae</i>	CBS 130.71 ^T	MT078884	MT078853	MT078918	MT078845
<i>Metarhizium flavoviride</i>	CBS 125.65	MT078885	MT078854	MT078919	MT078846
<i>Metarhizium flavoviride</i>	CBS 700.74	–	MT078855	MT078920	MT078847
<i>Metarhizium flavoviride</i>	CBS 218.56 ^T	MH857590	MH869139	KJ398694	KJ398787
<i>Moelleriella phyllogena</i>	CUP 067785	–	EU392610	–	EU392674
<i>Moelleriella phyllogena</i>	CUP 067793	–	EU392608	–	EU392672
<i>Moelleriella umbospora</i>	CUP 067817 ^T	–	EU392628	–	EU392688
<i>Morakotia fusca</i>	BCC 64125	–	KY794862	–	KY794857
<i>Morakotia fusca</i>	BCC 79272 ^T	–	KY794861	–	KY794856
<i>Mycophilomyces periconiae</i>	CPC 27558	NR_154209	NG_059746	–	–
<i>Myriogenospora atramentosa</i>	A.E.G 96-32	–	AY489733	DQ522455	AY489628
<i>Myrotheciomyces corymbiae</i>	CPC 33206	NR_160351	NG_064542	–	–

Species	Strain No.	GenBank Accession No.			
		ITS	LSU	<i>RPB2</i>	<i>tef-1α</i>
<i>Myrothecium inundatum</i>	IMI158855	–	AY489731	–	AY489626
<i>Myrothecium roridum</i>	ATCC 16297	–	AY489708	–	AY489603
<i>Myrothecium verrucaria</i>	ATCC 9095	–	AY489713	–	AY489608
<i>Nectria cinnabarina</i>	CBS 125165	HM484548	HM484562	KM232402	HM484527
<i>Nectria nigrescens</i>	CBS 125148	HM484707	HM484720	KM232403	HM484672
<i>Nectriopsis violacea</i>	CBS 424.64	–	AY489719	–	–
<i>Neoaraneomyces araneicola</i>	DY101711 ^T	MW730520	MW730609	MW753026	MW753033
<i>Neoaraneomyces araneicola</i>	DY101712	MW730522	MW730610	MW753027	MW753034
<i>Neobarya parasitica</i>	Marson s/n	KP899626	KP899626	–	–
<i>Neonectria candida</i>	CBS 151.29	JF735313	AY677333	–	JF735791
<i>Neonectria faginata</i>	CBS 217.67	HQ840385	HQ840382	DQ789797	JF268746
<i>Neonectria neomacrospora</i>	CBS 118984	HQ840388	HQ840379	DQ789810	JF268754
<i>Neonectria ramulariae</i>	CBS 182.36	HM054157	HM042435	DQ789793	HM054092
<i>Neurospora crassa</i>	ICMP 6360	AY681193	AY681158	–	–
<i>Niesslia exilis</i>	CBS 560.74	–	AY489720	–	AY489614
<i>Nigelia aurantiaca</i>	BCC13019	–	GU979948	GU979971	GU979957
<i>Nigelia martiale</i>	EFCC 6863	–	JF415974	–	JF416016
<i>Ophiocordyceps heteropoda</i>	EFCC 10125	JN049852	EF468812	EF468914	EF468752
<i>Ophiocordyceps sinensis</i>	EFCC 7287	JN049854	EF468827	EF468924	EF468767
<i>Ophiocordyceps stylophor</i>	OSC 111000	JN049828	DQ518766	DQ522433	DQ522337
<i>Orbiocrella petchii</i>	NHJ 6209	JN049861	EU369039	EU369081	EU369023
<i>Orbiocrella petchii</i>	NHJ 6240	–	EU369038	EU369082	EU369022
<i>Papiliomyces liangshanensis</i>	EFCC 1452	–	EF468815	–	EF468756
<i>Papiliomyces liangshanensis</i>	EFCC 1523	–	EF468814	EF468918	EF468755
<i>Papiliomyces shibinensis</i>	GZUH SB13050311 ^T	NR154178	–	–	KR153589
<i>Parametarhizium changbaiense</i>	CGMCC 19143 ^T	MN589741	MN589994	MT921829	MN908589
<i>Parametarhizium hingganense</i>	CGMCC 19144	MN055703	MN061635	MT939494	MN065770
<i>Paraneoaraneomyces sinensis</i>	ZY 22.006	OQ709254	OQ709260	OQ719621	OQ719626
<i>Paraneoaraneomyces sinensis</i>	ZY 22.007	OQ709255	OQ709261	OQ719622	OQ719627
<i>Paraneoaraneomyces sinensis</i>	ZY 22.008 ^T	OQ709256	OQ709262	OQ719623	OQ719629
<i>Parepichloe cinerea</i>	Ne-01	AB065425	–	–	–
<i>Peethambara spirostriata</i>	CBS110115	–	AY489724	EF692516	AY489619
<i>Periglandula ipomoeae</i>	IasaF13	–	–	KP689517	KP689568
<i>Pochonia boninensis</i>	JCM 18597	AB709858	AB709831	AB758693	AB758463
<i>Pochonia chlamydosporia</i>	CBS 101244	JN049821	DQ518758	DQ522424	DQ522327
<i>Pseudometarhizium araneogenum</i>	DY101741	MW730532	MW730618	MW753030	MW753037
<i>Pseudometarhizium araneogenum</i>	DY101742	MW730534	MW730619	MW753031	MW753038
<i>Pseudometarhizium lepidopterorum</i>	SD05361 ^T	MW730543	MW730624	–	MW753041
<i>Pseudometarhizium lepidopterorum</i>	SD05362	MW730611	MW730629	–	MW753042
<i>Purpureomyces maesotensis</i>	BCC 88441	MN781916	MN781877	MN781824	MN781734
<i>Purpureomyces maesotensis</i>	BCC 85349	MN781928	MN781872	–	MN781729
<i>Purpureomyces maesotensis</i>	BCC 89300 ^T	MN781917	MN781876	–	MN781733
<i>Regiocrella camerunensis</i>	ARSEF 7682	–	DQ118735	–	DQ118743
<i>Rosasphaeria moravica</i>	LMM	JF440985	–	JF440986	JF440987
<i>Rotiferophthora angustispora</i>	CBS 101437	AJ292412	AF339535	DQ522460	AF543776
<i>Roumegueriella rufula</i>	CBS 346.85	–	DQ518776	DQ522461	DQ522355
<i>Roumegueriella rufula</i>	GJS 91-164	–	EF469082	EF469116	EF469070
<i>Samuelsia chalalensis</i>	CUP 067856 ^T	–	EU392637	–	EU392691
<i>Samuelsia mundiveteris</i>	BCC 40021	–	GU552152	–	GU552145
<i>Samuelsia rufobrunnea</i>	CUP 067858 ^T	–	AY986918	–	AY986944
<i>Sarocladium bacillisporum</i>	CBS 425.67	NR_145039	MH870718	–	–
<i>Sarocladium dejongiae</i>	CBS 144929 ^T	NR_161153	NG_067854	–	–
<i>Sarocladium implicatum</i>	CBS 959.72 ^T	HG965023	MH878470	–	–
<i>Sarocladium subulatum</i>	CBS 217.35	MH855652	NG_070566	–	–

Species	Strain No.	GenBank Accession No.			
		ITS	LSU	<i>RPB2</i>	<i>tef-1α</i>
<i>Sarocladium terricola</i>	CBS 243.59	MH857853	MH869389	–	–
<i>Shimizuomyces paradoxus</i>	EFCC 6279	JN049847	EF469084	EF469117	EF469071
<i>Shimizuomyces paradoxus</i>	EFCC 6564	–	EF469083	EF469118	EF469072
<i>Simplicillium lamellicola</i>	CBS 116.25 ^T	AJ292393	MH866307	DQ522462	DQ522356
<i>Simplicillium lanosoniveum</i>	CBS 101267	AJ292395	–	DQ522463	DQ522357
<i>Simplicillium lanosoniveum</i>	CBS 704.86	AJ292396	AF339553	DQ522464	DQ522358
<i>Sordaria fimicola</i>	AFTOL-ID 216	DQ518178	–	–	DQ518175
<i>Stachybotrys eucylindrospora</i>	ATCC 18851	JN942887	JN938869	–	–
<i>Sphaerostilbella aureonitens</i>	GJS74-87	FJ442633	HM466683	FJ442763	–
<i>Sphaerostilbella berkeleyana</i>	GJS82-274	–	U00756	–	AF543783
<i>Sphaerostilbella chlorohalonata</i>	DAOM 235557	JN942888	JN938870	–	–
<i>Stachybotrys microspora</i>	CBS 186.79	–	–	DQ676580	DQ676604
<i>Stephanonectria keithii</i>	GJS92-133	–	AY489727	–	AY489622
<i>Sungia yongmunensis</i>	EFCC 2131 ^T	JN049856	EF468833	–	EF468770
<i>Sungia yongmunensis</i>	EFCC 2135	–	EF468834	–	EF468769
<i>Tilachlidium brachiatum</i>	CBS 506.67	KM231839	HQ232177	KM232415	KM231976
<i>Tilachlidium brachiatum</i>	CBS 363.97	KM231838	KM231719	KM232414	KM231975
<i>Tolypocladium inflatum</i>	SCALT1007-002	KC963032	–	–	–
<i>Trichoderma aggressivum</i>	CBS100525	–	JN939837	JQ014130	–
<i>Trichoderma viride</i>	GJS89-127	–	AY489726	–	AY489621
<i>Trichothecium roseum</i>	DUCC 502	JN937590	JX458860	–	–
<i>Tyrannicordyceps fraticida</i>	TNS-F 19011	JQ349068	JQ257023	JQ257021	JQ257028
<i>Ustilaginoidea dichromonae</i>	MRL IB9228	–	–	JQ257018	JQ257025
<i>Ustilaginoidea virens</i>	ATCC 16180	–	–	JQ257019	JQ257026
<i>Ustilaginoidea virens</i>	MAFF 240421	–	JQ257011	JQ257017	JQ257024
<i>Yosiokobayasia kusanagiensis</i>	TNS-F18494	–	JF415972	–	JF416014
<i>Pleurocordyceps aurantiaca</i>	MFLUCC 17-2113	MG136916	MG136910	MG136870	MG136875
<i>Pleurocordyceps marginaliradians</i>	MFLU 17-1582 ^T	MG136920	MG136914	MG271931	MG136878

Note: * J.F. White, Scale on Arundinaria tecta, North Carolina, 2000. New strains or species are in bold type.“^T” denotes ex-type. Abbreviations for collections: ARSEF, USDA-ARS Collection of Entomopathogenic Fungal cultures, Ithaca, NY; ATCC, American Type Culture Collection, USA; BCC, BIOTEC Culture Collection, KlongLuang, Thailand; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CGMCC, China General Microbiological Culture Collection Center, China; EFCC, Entomopathogenic Fungal Culture Collection, Chuncheon, Korea; GZUH, Guizhou University Herbarium, Guiyang, Guizhou, China; HMAS, Herbarium of Mycology, Chinese Academy of Sciences; MAFF, Ministry of Agriculture, Forestry and Fisheries of Japan, Tokyo, Japan; NHJ, Nigel Hywel-Jones personal collection; OSC, Oregon State University Herbarium, Corvallis, OR; TNS-F, the mycological herbarium of the National Museum of Nature and Science, Tsukuba, Ibaraki, Japan; WAC, Western Australian Plant Pathology Reference Culture Collection, Australia, Perth.

Sequence alignments and phylogenetic analyses

DNASTAR™ Lasergene (v 6.0) was used to edit DNA sequences in this study. The ITS, LSU, *RPB2* and *tef-1α* sequences were downloaded from GenBank, based on Sun et al. (2017), Chen et al. (2022a), Zhang et al. (2023), Zhu et al. (2023), and Tan and Shivas (2023, 2024) and others selected based on BLASTn searches in GenBank. ITS sequences and other loci were aligned and edited by MAFFT v.7.037b (Katoh and Standley 2013) and MEGA6 (Tamura et al. 2013). Combined sequences of ITS, LSU, *RPB2* and *tef-1α* were obtained using SequenceMatrix v.1.7.8 (Vaidya et al. 2011). The model was selected for Bayesian analysis by ModelFinder (Kalyaanamoorthy et al. 2017) in PhyloSuite (v1.2.2) software (Zhang et al. 2020). The combined dataset of ITS, LSU, *RPB2* and *tef-1α* sequence data (analysis 1 and analysis 2) was analyzed using Bayesian inference (BI) and maximum likelihood (ML) methods. For BI, a Markov chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities for the combined sequence datasets using MrBayes v.3.2 (Ronquist et al. 2012).

The Bayesian analysis resulted in 20,001 trees after 10,000,000 generations. The first 4,000 trees, representing the burn-in phase of the analysis, were discarded, while the remaining 16,001 trees were used to calculate posterior probabilities in the majority rule consensus tree. After the analysis was finished, each run was examined using the program Tracer v.1.5 (Drummond and Rambaut 2007) to determine burn-in and confirm that both runs had converged. ML analyses were constructed with IQ-TREE (v 2.0) (Trifinopoulos et al. 2016), using an automatic selection of the model according to BIC.

Results

Phylogenetic analyses

The phylogenetic tree (Fig. 1) of analysis 1 was generated to determine the family placement of the new strains and *Chlorocillium* species. *Gelasinospora tetrasperma* Dowding (AFTOL-ID 1287), *Neurospora crassa* Shear & B.O. Dodge (ICMP 6360) and *Sordaria fimicola* (Roberge ex Desm.) Ces. & De Not. (AFTOL-ID 216) were used as the outgroup taxa in analysis 1. The concatenated sequences of analysis 1 included 79 taxa and consisted of 3,126 (ITS, 633; LSU, 842; *RPB2*, 846; and *tef-1α*, 805) characters with gaps. The phylogenetic tree (Fig. 2) of analysis 2 was generated to determine the establishment of *Chlorocillium* species in Clavicipitaceae (Suppl. material 1). *Pleurocordyceps aurantiaca* (Y.P. Xiao, T.C. Wen & K.D. Hyde) Y.H. Wang, et al. (MFLUCC 17-2113) and *Pleurocordyceps marginaliradians* (Y.P. Xiao, T.C. Wen & K.D. Hyde) Y.H. Wang, et al. (MFLU 17-1582) were used as the outgroups in analysis 2. The concatenated sequences of analysis 2 included 69 taxa and consisted of 3,120 (ITS, 671; LSU, 740; *RPB2*, 825; and *tef-1α*, 884) characters with gaps.

Analysis 1: The selected model for ML analysis was TIM2+F+I+G4. The final value of the highest scoring tree was −46,827.254, which was obtained from an ML analysis of the dataset (ITS+LSU+*RPB2*+*tef-1α*). The parameters of the rate heterogeneity model used to analyze the dataset were estimated using the following frequencies: A = 0.236, C = 0.274, G = 0.278, T = 0.211; substitution rates AC = 1.44290, AG = 2.23422, AT = 1.44290, CG = 1.00000, CT = 5.74279 and GT = 1.00000, as well as the gamma distribution shape parameter α = 0.630. The selected models for BI analysis were GTR+F+I+G4 (ITS, LSU and *RPB2*), and GTR+F+G4 (*tef-1α*). The phylogenetic trees (Fig. 1) constructed using ML and BI analyses were largely congruent and strongly supported in most branches. The new strains DY05041 and DY05042 clustered into a group of the family Calcarisporiaceae and have a close relationship with *Calcarisporium arbuscula* (CBS 221.73 and CBS 900.68) and *C. yuanyangense* (YFCC 22099256). Strains KY07181, KY07182 and *Chlorocillium* species clustered as an independent clade and belong to the family Clavicipitaceae. Moreover, *Pseudometarhizium araneogenum* W.H. Chen, et al. (SD05361 and SD05362) and *P. lepidopterorum* W.H. Chen, et al. were grouped in *Chlorocillium* s. str. clade.

Analysis 2: The selected model for ML analysis was TN+F+I+G4. The final value of the highest scoring tree was −41,817.340, which was obtained from the ML analysis of the dataset (ITS+LSU+*RPB2*+*tef-1α*). The parameters of the GTR model used to analyze the dataset were estimated based on the following frequencies: A = 0.233, C = 0.280, G = 0.277, T = 0.210; substitution rates AC =

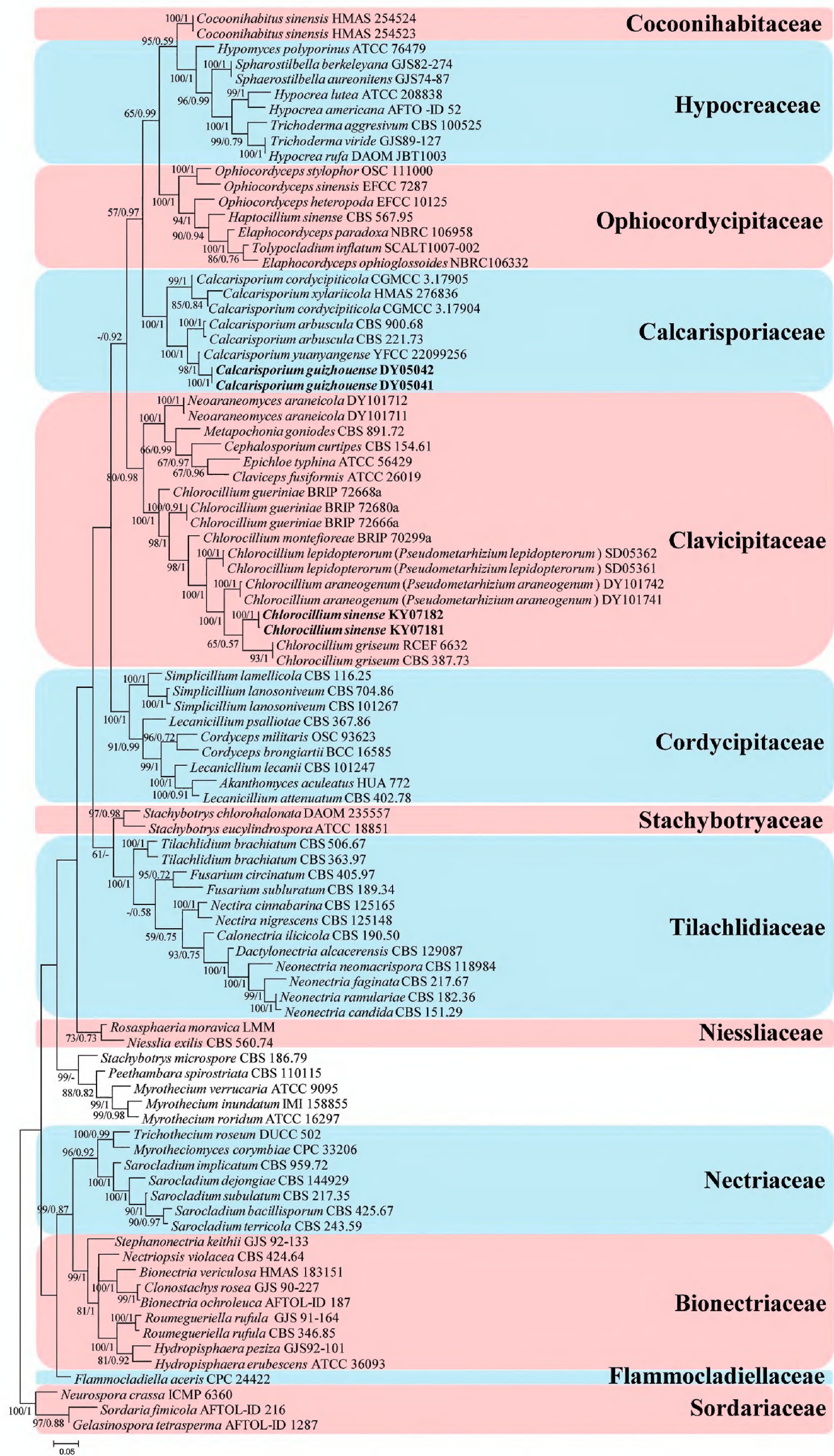


Figure 1. Phylogenetic analysis of the new strains and *Chlorocillium* species in the order Hypocreales based on multi-gene dataset (ITS, LSU, *RPB2* and *tef-1α*). Statistical support values ($\geq 50\%/0.5$) are shown at the nodes for ML bootstrap support/BI posterior probabilities. The new taxa are in bold.

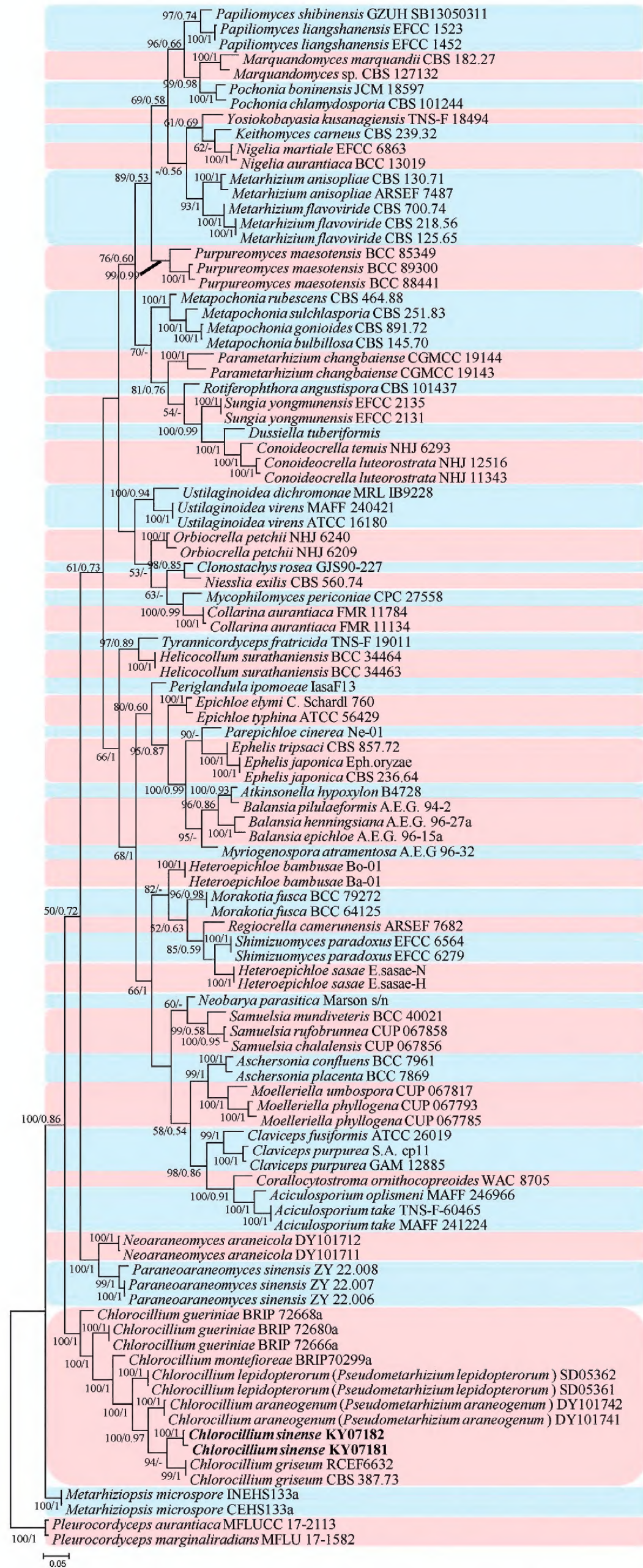


Figure 2. Phylogenetic analysis of *Chlorocillium* species in Clavicipitaceae, based on multigene dataset (ITS, LSU, *RPB2* and *tef-1α*). Statistical support values ($\geq 50\%/0.5$) are shown at the nodes for ML bootstrap support/BI posterior probabilities. The new taxa are in bold.

1.00000, AG = 2.55486, AT = 1.00000, CG = 1.00000, CT = 5.56065 and GT = 1.00000, as well as the gamma distribution shape parameter $\alpha = 0.442$. The selected models for BI analysis were GTR+F+I+G4 (ITS+LSU+ *tef*-1 α) and SYM+G4 (*RPB2*). The phylogenetic trees (Fig. 2) constructed using ML and BI analyses were largely congruent and strongly supported in most branches. Most genera clustered into independent clades. Strains KY07181 and KY07182 clustered into an independent clade with close relationship with *Chlorocillium griseum*. However, *Pseudometarhizium* species clustered within the genus *Chlorocillium*.

Taxonomy

***Calcarisporium guizhouense* W.H. Chen, Y.F. Han & J.D. Liang, sp. nov.**

MycoBank No: 854035

Fig. 3

Etymology. Referring to its type location in Guizhou Province.

Type. CHINA • Guizhou Province, Qiannan Buyei and Miao Autonomous Prefecture, Duyun City, Mayao River Valley (26°22'8.3748"N, 107°23'16.96"E), on *Cordyceps* sp., 1 May 2022, Wanhao Chen, GZAC DY0504 (holotype), ex-type DY05041.

Description. Colonies on PDA attaining a diameter of 28–29 mm after 14 days at 25 °C, white, consisting of a basal felt, floccose hyphal overgrowth, yellowish-white; reverse light brown to brown. Hyphae septate, hyaline, smooth-walled, 2.0–2.2 μm wide. Conidiophores erect, hyaline, verticillately branched, with 1–3 conidiogenous cells. Conidiogenous cells 15.6–23.2 $\times$ 1.5–1.7 μm , hyaline, cylindrical at base, gradually tapering near the apex, holoblastic to polyblastic, sympodial, apically with a cluster of conidium-bearing denticles. Conidia 5.2–8.6 $\times$ 1.8–2.2 μm , hyaline, smooth-walled, thin-walled, cylindrical, unicellular, acuminate.

Substrate. *Cordyceps* sp.

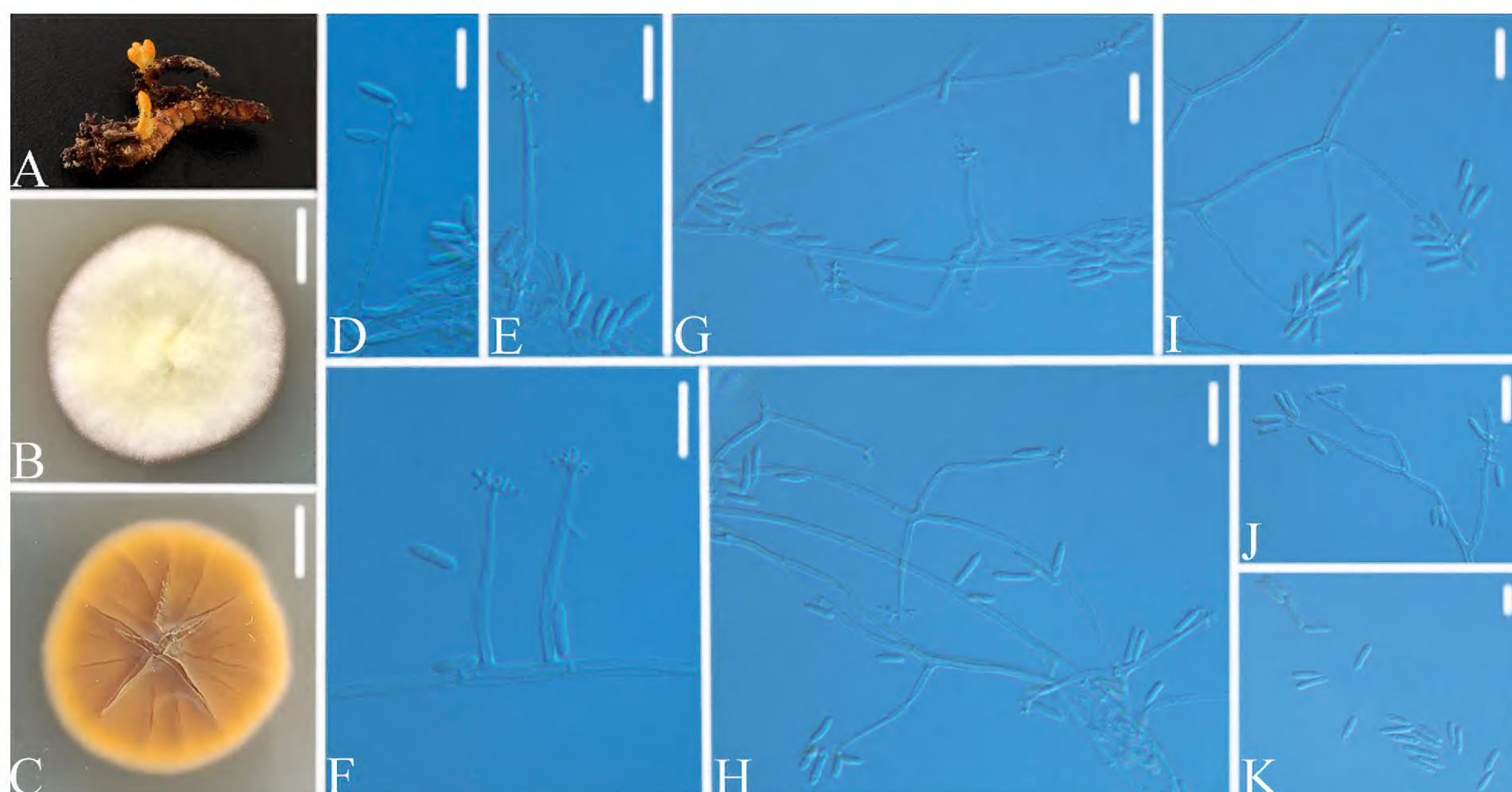


Figure 3. *Calcarisporium guizhouense* **A** the substrate *Cordyceps* sp. (fungicolous) **B, C** PDA culture plate showing top (**B**) and reverse (**C**) sides of the colony **D–K** Conidiogenous cells and conidia. Scale bars: 10 mm (**B, C**); 10 μm (**D–K**).

Additional strain examined. CHINA • Guizhou Province, Qiannan Buyei and Miao Autonomous Prefecture, Duyun City, Mayao River Valley (26°22'8.3748"N, 107°23'16.96"E). On *Cordyceps* sp., 1 May 2022, Wanhao Chen, DY05042 (living culture).

Notes. *Calcarisporium guizhouense* was easily identified as *Calcarisporium*, based on the BLASTn result in NCBI and its verticillate conidiophores and sympodial conidiation. Phylogenetic analyses show that *Calcarisporium guizhouense* has close relationships to *C. arbuscula* and *C. yuanyangense* (Fig. 1). However, *C. guizhouense* was easily distinguished from *C. arbuscula* (Conidia: ovoid to ellipsoid, 4–11 × 1.8–3.3 µm; substrate: decaying agaric) and *C. yuanyangense* (Conidia: ovoid to ellipsoid, 6.33–9.68 × 1.87–2.63 µm; substrate: *Ophiocordyceps nutans*) by its smaller cylindrical conidia and the substrate. Thus, the morphological characteristics and molecular phylogenetic results support *C. guizhouense* as a new species.

***Chlorocillium* Zare & W. Gams, Mycol. Progr. 15: 1005, 2016**

= *Pseudometarhizium* W.H. Chen, Y.F. Han, J.D. Liang & Z.Q. Liang, MycoKeys 91: 59, 2022 MycoBank No: 842641.

Type species. *Chlorocillium griseum* (Petch) Zare & W. Gams.

Notes. Chen et al. (2022a) introduced the genus *Pseudometarhizium* with *P. araneogenum* (type species) and *P. lepidopterorum* in the family Clavicipitaceae. However, in the present study, the multi-gene phylogenetic analyses revealed that *Pseudometarhizium* species clustered with *Chlorocillium* s. str. (Fig. 2). Besides, the typical characteristics of *Pseudometarhizium* are entirely consistent with *Chlorocillium*. Thus, the genus *Pseudometarhizium* is synonymized under *Chlorocillium* and the two known species of *Pseudometarhizium* are transferred into *Chlorocillium*.

***Chlorocillium sinense* W.H. Chen, Y.F. Han & J.D. Liang, sp. nov.**

MycoBank No: 853937

Fig. 4

Etymology. Referring to the country where the fungus was first discovered.

Type. CHINA • Guizhou Province, Guiyang, Kaiyang County, Monkey-Ear Tiankeng (27°5'12.138"N, 107°0'48.42"E), on a dead spider (Araneae), 19 July 2023, Wanhao Chen, GZAC KY0718 (holotype), ex-type, KY07181.

Description. Colonies on PDA reaching 15–17 mm in diameter in 14 days at 25 °C, green to yellowish green in center with white margin, reverse yellowish to light brown. Hyphae septate, hyaline, smooth-walled, 1.3–2.1 µm wide. Conidiophores hyaline, smooth-walled, emerging from aerial hyphae or chondroid mycelium, with single phialide or whorls of 2–4 phialides or verticillium-like from hyphae directly. Phialides cylindrical, somewhat inflated base, 11.7–20.1 × 1.1–1.3 µm, tapering to a thin neck. Conidia hyaline, smooth-walled, fusiform to ellipsoidal, 1.9–2.9 × 0.8–1.2 µm, forming divergent and basipetal chains. Octahedral crystals and chlamydospores absent.

Host. Spider (Araneae).

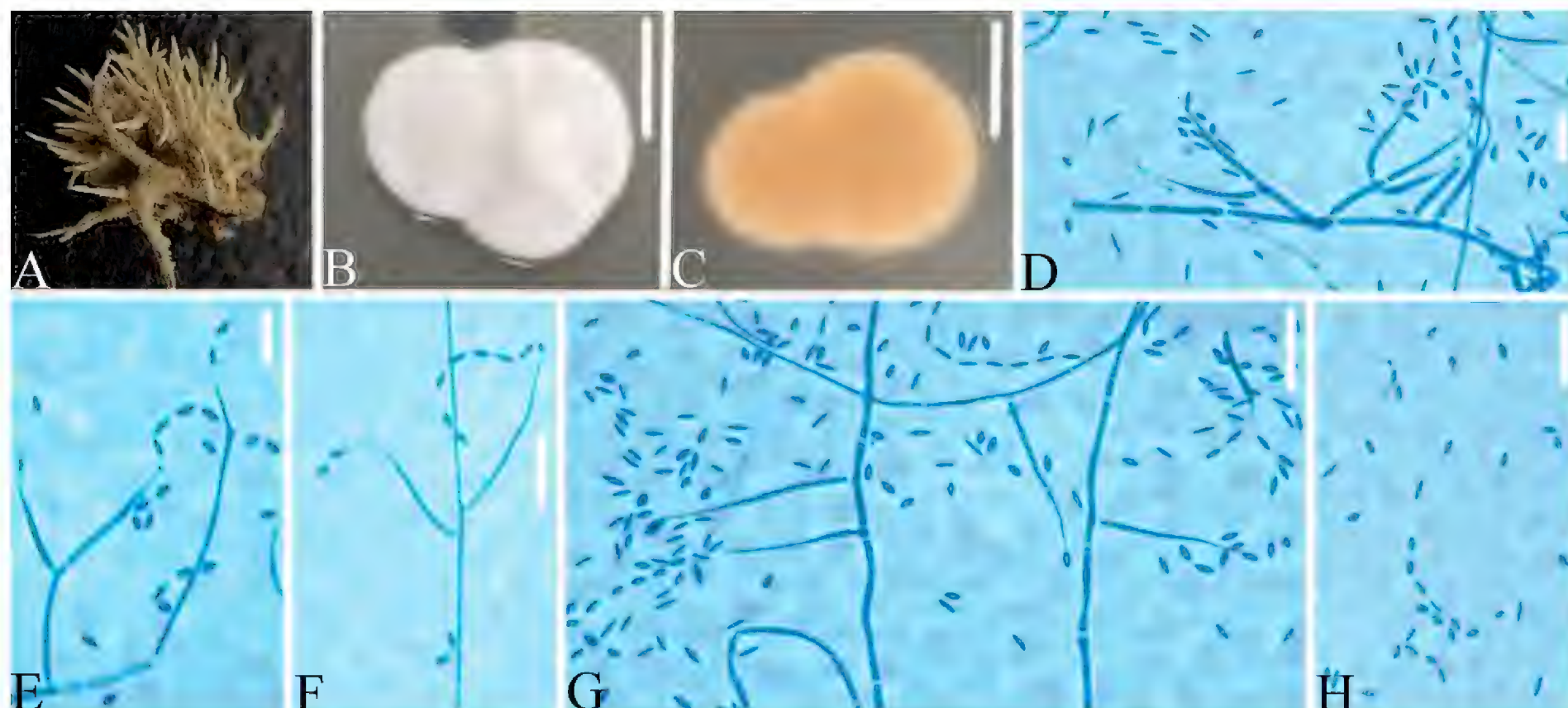


Figure 4. *Chlorocillium sinense* **A** infected spider **B, C** PDA-containing culture plate showing **B** the front and **C** reverse sides of the colony **D–H** phialides, conidia in chains and conidia. Scale bars: 10mm (**B, C**); 10 μ m (**D–H**).

Additional strain examined. CHINA • Guizhou Province, Guiyang, Kaiyang County, Monkey-Ear Tiankeng (27°5'12.138"N, 107°0'48.42"E). On a dead spider (Araneae), 19 July 2023, Wanhao Chen, KY07182 (living culture).

Remarks. *Chlorocillium sinense* was easily identified as *Chlorocillium*, based on the BLASTn result in NCBI. The phylogenetic analysis of the combined data-set of ITS, LSU, *RPB2* and *tef-1 α* sequence data showed that the new collections clustered as an independent clade with close relationship to *C. araneogenum*, *C. griseum* and *C. lepidopterorum* (Fig. 2). Table 2 provides the different morphological characteristics that can be used to differentiate *Chlorocillium sinense* from other phylogenetically close species.

New combinations

***Chlorocillium araneogenum* (W.H. Chen, Y.F. Han, J.D. Liang & Z.Q. Liang)**

W.H. Chen, Y.F. Han & J.D. Liang, comb. nov.

MycoBank No.: 853934

Pseudometarhizium araneogenum W.H. Chen, Y.F. Han, J.D. Liang & Z.Q. Liang, MycoKeys 91: 60 (2022) (Basionym).

Description. Colonies irregular on PDA, 1.8–2.8 cm diam. after 14 d at 25 °C, white, consisting of a basal felt, floccose hyphal overgrowth, reverse yellowish to pale brown or green. Prostrate hyphae smooth, septate, hyaline, 1.0–1.2 μ m diam. Erect conidiophores usually arising from aerial hyphae. Phialides solitary or in groups of two, 8.3–23.3 $\times$ 1.3–2.2 μ m, with a cylindrical basal portion, tapering into a short distinct neck. Conidia in chains, hyaline, fusiform, one-celled, 3.4–5.8 $\times$ 1.4–1.8 μ m.

Material examined (type material). CHINA • Duyun City, Qiannan Buyi and Miao Autonomous Prefecture, Guizhou Province. On a dead spider (Araneae), 1 October 2019, Wanhao Chen, GZAC DY10174, living cultures, DY101741, DY101742.

Table 2. Morphological comparison of the new species with other *Chlorocillium* species.

Species	Strain	Phialides (µm)	Conidia (µm)	Host	Octahedral crystals
<i>C. araneogenum</i>	DY101801	8.3–23.3 × 1.3–2.2	fusiform, 3.4–5.8 × 1.4–1.8	spider	absent
<i>C. griseum</i>	CBS 387.73	18–40(–55) × 2–2.5	fusiform, 4.5–6 × 1.0–1.5	spider	present
<i>C. lepidopterorum</i>	SD05361	21.2–33.7 × 1.1–1.4	fusiform, 3.1–4.3 × 1.3–1.5	pupa	absent
<i>C. sinense</i>	KY07181	11.7–20.1 × 1.1–1.3	fusiform to ellipsoidal, 1.9–2.9 × 0.8–1.2	spider	absent

***Chlorocillium lepidopterorum* (W.H. Chen, Y.F. Han, J.D. Liang & Z.Q. Liang)**
W.H. Chen, Y.F. Han & J.D. Liang, comb. nov.
MycoBank No.: 853935

Pseudometarhizium lepidopterorum W.H. Chen, Y.F. Han, J.D. Liang & Z.Q. Liang, MycoKeys 91: 60 (2022) (Basionym).

Description. Colonies on PDA, 1.4–2.0 cm diam. after 14 d at 25 °C, white, consisting of a basal felt and cottony, floccose hyphal overgrowth, reverse yellowish to pale green. Prostrate hyphae smooth, septate, hyaline, 1.0–2.0 µm diam. Erect conidiophores usually arising from aerial hyphae. Phialides solitary or in groups of two to three, 21.2–33.7 × 1.1–1.4 µm, with a cylindrical basal portion, tapering into a short distinct neck. Conidia in chains, hyaline, fusiform, one-celled, 3.1–4.3 × 1.3–1.5 µm.

Material examined (type material). CHINA • Sandu County, Qiannan Buyi and Miao Autonomous Prefecture, Guizhou Province, . On a pupa (Lepidoptera), 1 May 2019, Wanhao Chen, GZAC SD0536, living cultures, SD05361, SD05362.

Discussion

The complex terrain, mild climate, abundant rainfall, wide vegetation coverage, and diverse forest types and components, result in Guizhou Province having abundant resources of fungi associated with insects. However, while fungal species associated with insects are often found in forest and grassland reservations (Chen et al. 2019, 2020, Peng et al. 2023, Xiao et al. 2023, 2024, Liu et al. 2024); they are rarely found in special karst eco-environments, such as Tiankeng and valleys. Chen et al. (2022a, b, 2023) introduced thirteen new species associated with insects or spiders from Tiankeng or valleys.

Calcarisporium species are often reported as fungicolous and produce abundant secondary metabolites (Sun et al. 2017, Zhu et al. 2023). Three species, *C. cordycipiticola*, *C. ovalisporum* and *C. yuanyangense* were introduced from the entomopathogenic taxa, *Cordyceps militaris* (L.) Fr., *Hirsutella citriformis* Speare and *Ophiocordyceps nutans* (Pat.) G.H. Sung et al. In the present study, *Calcarisporium guizhouense* were introduced with the substrate *Cordyceps* sp. from Mayao River Valley.

Zare and Gams (2016) introduced the genus *Chlorocillium* with *C. griseum*, and the placement of the genus was not confirmed in Hypocreomycetidae. Wang (2022) noted that the genus *Chlorocillium* belongs to the order Hypocreales and has a close relationship with the family Clavicipitaceae. Tan and Shivas (2023, 2024) reported two new *Chlorocillium* species in Australia. However, the taxonomic status of the genus was still unclear.

In the order-level phylogenetic tree (Fig. 2), *Chlorocillium* species clustered into Clavicipitaceae (Hypocreales, Hypocreomycetidae, Sordariomycetes). Thus, the combined dataset of ITS, LSU, *RPB2*, and *tef-1α* showed the taxonomic placement of *Chlorocillium* in Clavicipitaceae for the first time. Furthermore, in the family-level phylogenetic tree (Fig. 1), strains KY07181 and KY07182 formed an independent branch and clustered with *C. griseum*, *C. gueriniae*, *C. montefioreae*, *P. araneogenum* and *P. lepidopterorum* in a subclade. *Pseudometarhizium* species were also consistent with the genus *Chlorocillium* based on the typical characteristics. Thus, we synonymized *Pseudometarhizium* under *Chlorocillium* and transferred its species.

Conclusion

Two new species, *Chlorocillium sinense* and *Calcarisporium guizhouense*, were established and described in the present study. The genus *Chlorocillium* was confirmed in the family Clavicipitaceae. Furthermore, the genus *Pseudometarhizium* was synonymized with *Chlorocillium* and its species were transferred.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Data curation: WHC. Formal analysis: DL, JDL, WHC. Funding acquisition: JHZ, WHC, YFH, XXR. Resources: DL, WHC. Writing – original draft: DL, WHC. Writing – review and editing: XXR, JHZ, JDL, YFH.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

The genus contain in the family Clavicipitaceae

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